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Interactions of Indoles with Specific Binding Sites for 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Rat Liver

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SUMMARY

In order to identify some of the structural requirements for binding of indoles to the receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), we have investigated the capacity of various indoles to inhibit specific [1,6-^{3}H]TCDD binding in rat liver cytosol, as analyzed by electrofocusing in polyacrylamide gel. Of these indoles, indolo[3,2-*b*]carbazole was the most active. The IC_{50} value for receptor binding of indolo[3,2-*b*]carbazole as well as for 2,3,7,8-tetrachlorodibenzofuran was 3.6 nM, whereas that of 5,6-benzoflavone was 26 nM. Both indolo[3,2-*b*]carbazole and 2,3,7,8-tetrachlorodibenzofuran competitively inhibited the binding of [^{3}H]TCDD to the receptor. The well-known microsomal enzyme inducer 3,3′-diindolylmethane did not interact significantly with the TCDD receptor. Previous concepts of structure-activity relationships for binding of chlorinated dioxins to the TCDD receptor fail to account for the receptor binding of unhalogenated aryl hydrocarbon hydroxylase inducers such as 5,6-benzoflavone. We have instead considered the true three-dimensional space occupied by some receptor ligands by means of a computer using crystallographic data as inputs. When the atomic van der Waals radii were included, all potent receptor ligands studied could be fitted into a rectangle of 6.8 × 13.7 Å.

INTRODUCTION

Many environmental carcinogenic PAH[2] are substrates for certain inducible microsomal enzyme activities (1). Some flavonoids and indoles have been reported to induce certain microsomal enzyme activities and to protect against benzo[*a*]pyrene-induced neoplasia in rats (2). This protection has been suggested to involve inactivation of carcinogens by the induced enzymes, before the carcinogens reach their target tissues (2).

AHH is one of the microsomal enzyme activities which is induced in the liver and intestine by indoles (2). In the rat liver, AHH is constituted by several isozymes of cytochrome P-450 (3), some of which are inducible by certain PAH, such as 3-methylcholanthrene and 5,6-benzoflavone (3). When induced, one of these particular isozymes is responsible for most of the AHH activity (3). This induction is thought to be mediated by binding of the inducer to a soluble receptor protein with a high affinity for TCDD (4), the so-called TCDD receptor.

We have previously reported that the most potent microsomal enzyme inducer identified in cruciferous plants, indole-3-carbinol, as well as quercetin pentamethylether, a flavonoid that also induces AHH, have very low receptor-binding affinities (5). According to the mechanism mentioned above, AHH inducers are expected to bind to the TCDD receptor. In order to clarify this apparent inconsistency, we have synthesized or obtained a number of indoles and examined their interaction with the specific [^{3}H]TCDD-binding sites in rat liver.

MATERIALS AND METHODS

Chemicals. 5,6-Benzoflavone and indole-3-acetonitrile were purchased from Aldrich-Europe (Beerse, Belgium). 2-Hydroxyethyl mercaptan, hemoglobin (beef blood), ferritin (horse spleen), charcoal (Norit A), benzo[*a*]pyrene, indole-3-acetic acid, indole-3-carboxaldehyde,

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[2] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; AHH, aryl hydrocarbon hydroxylase activity; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; IC_{50}, inhibitory concentration which competes for one-half of the specific binding sites. Indolo[3,2-*b*]carbazole and indolo[2,3-*a*]carbazole should, according to Chemical Abstracts, be called 5,11-dihydroindolo[3,2-*b*]carbazole and 11,12-dihydroindolo[2,3-*a*]indolocarbazole, respectively.

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tryptamine, and L-tryptophan were obtained from Sigma. 7,8-Benzoflavone and indole-3-carbinol were from EGA-Chemie (Steinheim, West Germany). TCDBF and [1,6-^{3}H]TCDD (specific activity 28.9 Ci/mmol) were kind gifts from Drs. C. Rappe, University of Umeå (Umeå, Sweden) and A. Poland, McArdle Laboratory for Cancer Research (Madison, WI), respectively. The [^{3}H]TCDD was stored at 2–4° in toluene/ethanol (4:1, v/v) in the dark. 3,3′,4,4′-Tetrachlorobiphenyl was generously provided by Dr. Åke Bergman, Wallenberg Laboratory, University of Stockholm (Stockholm, Sweden). Dextran T-70 was purchased from Pharmacia Fine Chemicals. Glycerol, K_2HPO_4, KH_2PO_4, indole, and dimethyl sulfoxide (Uvasol grade) were obtained from Merck (Darmstadt, West Germany). EDTA was obtained from Fluka AG (Buchs SG, West Germany). Trypsin-tosylphenylalanyl chloromethyl ketone (269 units/mg) was purchased from Worthington. Scintillation fluid (Scintillator 299) was from Packard Instrument Co. Polyacrylamide gels for electrofocusing (LKB PAG-Plates, pH range 3.5–9.5) were purchased from LKB-produkter AB (Bromma, Sweden). These chemicals were used without further purification.

Indolo[3,2-*b*]carbazole was made from 3,3′-diindolylmethane as described by Bergman (6). The crude product was purified by sublimation under reduced pressure, and its purity was at least 99.5% as judged by gas chromatography-mass spectrometry. The indoles mentioned below were at least 95% pure. Ellipticine (7), indolo[2,3-*a*]carbazole (8), *5H*-benzo[*b*]carbazole (9), *6H*-indolo[2,3-*b*]quinoxaline (10), compound IV in Fig. 1 (11), 3,3′-diindolylmethane (12), 2,2′-dimethyl-3,3′-diindolylmethane (12), ascorbigen (13), indole-2-carbinol (14), and indole-3-ethanol (15) were synthesized as described in the references cited.

Animals. Eight-week-old male Sprague-Dawley rats obtained from A-Lab (Stockholm, Sweden) were used in all experiments. They were fed a standard pellet diet (Astra-Ewos R3, Astra, Södertälje, Sweden) *ad libitum* and had free access to tap water.

Buffer. The buffer used consisted of 20 mM phosphate, pH 7.2, 1 mM Na_2EDTA, 10% (w/v) glycerol, and 2 mM 2-hydroxyethylmercaptane.

Preparation of cytosol. The rats were killed by cervical dislocation and the liver was perfused *in situ* via the portal vein with 60 ml of ice-cold buffer. The liver was gently massaged during perfusion to ensure an even and thorough removal of the blood. The liver was then removed onto ice, and all further work was carried out at 2–4°. The liver was finely minced with scissors in 24 ml of buffer. The mince was homogenized in a Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 140,000 × g_{av} for 45 min in a fixed-angle 50 rotor in a Beckman L-8 M ultracentrifuge. The turbid lipid layer was carefully removed, and the clear supernatant was used as cytosol after dilution to 3.5 $A_{280\text{–}310\ nm}$/ml (corresponding to 2–4 mg of protein/ml, as determined by the method of Lowry *et al.* (16)).

In vitro labeling of cytosol. A 300 nM [^{3}H]TCDD solution in dimethyl sulfoxide was prepared. To each 1-ml aliquot of cytosol in borosilicate glass tubes, 2–10 μl of the [^{3}H]TCDD solution was added. Dimethyl sulfoxide was also used as solvent for unlabeled competitors. When necessary, dimethyl sulfoxide was added to keep the total solvent concentration constant in all incubations of one experiment. [^{3}H]TCDD was always added as the final step. The amount of nonspecific binding was monitored in all experiments with an incubation containing 1.5 nM [^{3}H]TCDD and 150 nM TCDBF.

To estimate their binding affinity for the TCDD receptor, potential ligands were first incubated at 150 or 1500 nM concentrations in the presence of 1.5 nM [^{3}H]TCDD. Ligands that at 150 nM inhibited more than 50% of the specific [^{3}H]TCDD binding were then, in three separate experiments, also incubated at six further concentrations giving inhibition of specific [^{3}H]TCDD binding of between 10 and 90%. Except for chlorinated compounds, new solutions of competitor were prepared for each of these three experiments. In all such experiments, an incubation with 1.5 nM [^{3}H]TCDD without competitor was used to determine total binding, which was used for calculation of maximum specific binding.

In order to ascertain that the inhibition of specific [^{3}H]TCDD binding caused by indolo[3,2-*b*]carbazole and TCDBF was competitive, these ligands were added at a fixed final concentration to six incubations to which [^{3}H]TCDD was added to yield final concentrations ranging from 0.2–3.0 nM.

Limited proteolysis of the receptor. After 2 h at 2°, duplicate 0.3-ml portions were removed from each incubation mixture for determination of radioactivity and calculation of the total [^{3}H]TCDD concentration. The remainder was incubated with 0.5 μg of trypsin/$A_{280\text{–}310\ nm}$ at 10° for 30 min (5). Trypsin was dissolved in water, and 5 μl of the solution was added to each incubation.

Dextran-coated charcoal treatment. After limited proteolysis, unbound [^{3}H]TCDD was removed from the trypsinized labeled cytosol by treatment of the remaining 0.4 ml with 0.3 ml of a dextran-coated charcoal solution (0.41% (w/v) Norit A and 0.041% (w/v) dextran T-70 in buffer) and left for 10 min at 2°. After centrifugation for 10 min at 2500 × *g*, aliquots of the resulting supernatant were assayed for radioactivity, and 0.3 ml was analyzed by electrofocusing.

Electrofocusing. Ready-made PAG plates were prefocused for 30 min using an LKB 2117 Multiphor instrument. The maximum settings/plate on an LKB 2197 power supply were 20 mA, 1500 V, 25 W. An LKB 2209 Multitemp cryothermostat pumped ethanol (at −2°) through the cooling plate. The samples were applied in acrylic plastic frames (inner dimension 7 × 15 mm; height 3 mm) placed on the gel 1 cm from the cathode electrode strip. Eight samples were analyzed simultaneously with hemoglobin and ferritin applied as standards between each sample frame. The electrofocusing was carried out for 90 min at 25 mA, 1500 V, 25 W. The pH was measured at 2° in the middle of the gel at 1-cm intervals after completed electrofocusing, using a surface pH electrode (type 403-30, Ingold, Zürich, Switzerland). In front of each sample frame, 4-cm long and 1.5-cm wide sections were cut from the gel and sliced into ten 4-mm sections with a pair of scissors. The slices were placed in plastic scintillation vials.

Liquid scintillation counting. Each slice was extracted by submersion in 5 ml of scintillation fluid and left in the dark at 2° for 24 h. The vials were counted in an LKB-Wallac Rackbeta 1516 liquid scintillation spectrometer with direct calculation of disintegrations/min via the external standard technique. The counting efficiency was on the average 40% under these conditions.

Quantification of specific [^{3}H]TCDD binding. The total binding of [^{3}H]TCDD in the cytosol was quantitated as the area below the radioactivity peak at pH 5.2, the apparent isoelectric point of the TCDD receptor (5), following electrofocusing of the incubation mixtures. The nonspecific binding was calculated and subtracted from the total binding in each incubation to obtain the specific binding, as recommended by Chamness and McGuire for analysis of steroid hormone-receptor-binding data (17). A specific binding of 50–70 fmol/mg of cytosolic protein was routinely observed.

Molecular structure studies. The coordinates used to plot molecular structures of compounds studied were obtained with three different methods. In all methods the view coordinate file was created from a Vector General 3404 vector-display unit driven by a VAX 11/750 computer running the FRODO (18) package program. This interactive program, mainly designed for protein model building, was used (second and third method) to model and build small molecules.

The first method was used when crystallographic coordinates were available, *i.e.*, for TCDD (19), 5,6-benzoflavone (20), 7,8-benzoflavone (20), and benzo[*a*]pyrene (21). These coordinates, given in fractions of the crystallographic cell dimensions, were transformed into Ångström (Å) units and orthogonalized.

The second method was used when molecular coordinates from crystallographic data were not published for the actual compound but could be derived in part from an existing structure. For the two indolocarbazoles, the indole trimer, 14-acetyldiindolo[2,3-*a*:2′,3′-*c*]carbazole (22), was used as the parent compound. The hydrogens were not present in the published structure and were added in standard positions using the display. This structure was then used to build indolo[3,2-*b*]carbazole and indolo[2,3-*a*]carbazole by removal of the exceeding at-

oms, transpositions, and rotations of the indole fragments, to obtain the final structure. The tetrachloro-biphenyl structure was derived from that of 1,1-dichloro-biphenyl 923), by replacement of the four corresponding hydrogens by chlorines, and the two chlorines by hydrogens. An angle of 40° was set between the two rings as indicated by crystallographic data and calculations (23). The views of both of the two possible conformers were then chosen for plotting.

For other compounds no coordinates were readily available, and in these cases the third method was used. Models of molecules were then built from bond and angle values using the program TOM[3] derived from FRODO (18). The bond length and angle values were, if not published for the actual compound, standard values taken from Pauling (24).

The views for plotting were chosen through the display. The coordinates obtained from the display were transformed to be accepted by the PLUTO[4] plotting program. From two sets of commands, ball and stick figures were drawn superimposed by a Hewlett-Packard 7220A plotter. The van der Waals radii values for the balls were taken from Pauling (24), *i.e.*, in Å: H, 1.2; O, 1.4; N, 1.5; Cl, 1.8; C, 1.7.

RESULTS

To identify some of the structural requirements for TCDD receptor binding of indoles that may induce AHH, we have synthesized or obtained various indoles and studied their receptor affinity. IC_{50} values of different compounds for their inhibition of specific [^{3}H]TCDD binding in rat liver cytosol are listed in Table 1, and the structural formulas of some of these compounds are shown in Fig. 1. Certain compounds lacking the indole nucleus were also included in this study for reference purposes (not shown in Fig. 1).

TABLE 1

Binding affinities of various competitors for the specific [^{3}H]TCDD-binding sites in rat liver cytosol

Assays were performed as described under "Materials and Methods," and IC_{50} values were calculated with the logit function as described earlier (5). Correlation coefficients for linear regression were generally above 0.95 for compounds with IC_{50} values below 150 nM. IC_{50} values for inhibition of specific [^{3}H]TCDD binding represent the mean ± SD from three separate experiments. Roman numerals refer to Fig. 1 for structures of the compounds and capital letters to Fig. 3 for projections plotted.

Competitor	IC_{50}
	nM
2,3,7,8-Tetrachlorodibenzofuran	3.6 ± 0.2
Indolo[3,2-*b*]carbazole (I) (C)	3.6 ± 2.6
5,6-Benzoflavone (D)	25.9 ± 12.6
Benzo[*a*]pyrene (B)	42.4 ± 9.6
5H-Benzo[2,3-*a*]carbazole (II)	69.6 ± 26.2
3,3′,4,4′-Tetrachlorobiphenyl (G, H)	<150
Indolo[2,3-*a*]carbazole (E) (III)	>150
Compound IV (IV)	>150
7,8-Benzoflavone (F)	>150
6H-Indolo[2,3-*b*]quinoxaline (V)	>150
Ellipticine (VI)	>1500
3,3′-Diindolylmethane (VII; R=H)	>1500
2,2′-Dimethyl-3,3′-diindolylmethane (VII; R=CH_3)	>1500
Tryptophan (VIII; X=CH_2—CH(NH$_2$)—COOH)	>1500
Tryptamine (X=$CH_2CH_2NH_2$)	>1500
Indole-3-carbinol (X=CH_2OH)	>1500
Indole-3-ethanol (X=CH_2CH_2OH)	>1500
Indole-3-acetic acid (X=CH_2COOH)	>1500
Indole-3-acetonitrile (X=CH_2CN)	>1500
Ascorbigen (X=CH_2—vitamin C)	>1500
Indole-2-carbinol	>1500

[3] Program available from C. Cambillau, E. Horjales, and T. A. Jones.

[4] Program available from Sam Motherwall (Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, England).

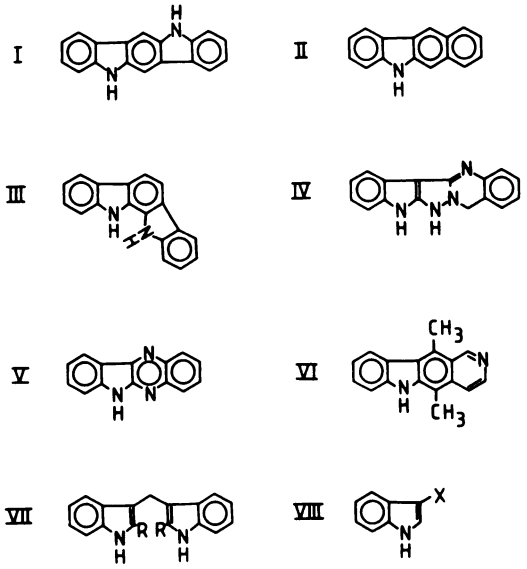

FIG. 1. *Structural formulas for some of the competitors used to displace [^{3}H]TCDD from its specific binding sites in rat liver cytosol*

Structures are shown for indolo[3,2-*b*]carbazole (I), *5H*-benzo[2,3-*a*]carbazole (II), indolo[2,3-*a*]carbazole (III), compound IV, *6H*-indolo[2,3-*b*]quinoxaline (V), ellipticine (VI), 3,3′-diindolylmethane (VII; R=H), 3-substituted indoles (VII).

The most potent ligand found was indolo[3,2-*b*]carbazole (I in Fig. 1; IC_{50} 3.6 ± 2.6 nM), which may be chemically derived (6) from 3,3′-diindolylmethane, described by Loub *et al.* (25) as one of the most potent inducers of AHH occurring in brussels sprouts. Indolo[2,3-*a*]carbazole (III; IC_{50} > 150 nM), an angular isomer of indolo[3,2-*b*]carbazole, was a much weaker ligand than indolo[3,2-*b*]carbazole, which may indicate that a relatively linear ring system is essential for high-affinity TCDD receptor binding of indoles. A corresponding difference in receptor affinity was observed when the linear (20) 5,6-benzoflavone (IC_{50} 25.9 ± 12.6 nM) was compared to 7,8-benzoflavone (IC_{50} > 150 nM), which is nonlinear (20).

Since indolocarbazoles (22) and benzoflavones (20) are plantar, the relatively high affinity of indolo[3,2-*b*]carbazole and 5,6-benzoflavone for the TCDD receptor is in line with earlier studies indicating planarity as a requirement for receptor ligands (4).

An opening of the ring system of the indolocarbazoles, leading to diindolylmethanes (*e.g.*, VII, in which R=H or R=CH_3; IC_{50} > 1500 nM), greatly reduced receptor affinity, perhaps due to loss of the rigid flat structure of the indolocarbazoles in these analogues. Similarly, chlorinated biphenyls, which have quite a flexible structure (23), bind to the TCDD receptor with considerably lower affinity (4) than the corresponding chlorinated dioxins with their rigid flat structure (19).

The fact that indoles smaller than indolo[3,2-*b*]carbazole (I), such as *5H*-benzocarbazole (II; IC_{50} 69.6 ± 26.2 nM), *6H*-indolo[2,3-*b*]quinoxaline (V; IC_{50} > 150 nM), and ellipticine (VI; IC_{50} > 150 nM) have lower affinity for the TCDD receptor than indolo[3,2-*b*]carbazole may indicate that a minimal molecular size is required for high-affinity receptor binding. The low receptor affinity of compound IV (IC_{50} > 150 nM) might be due to its nonlinearity. The lower binding affinities of these latter four indoles seem unlikely to be due to electrostatic effects, since none of these aromatic nitrogens should be protonated at a pH of 7.2 (26). Indoles substituted at the 3-position (with the general structure VIII), and indole-2-carbinol failed to inhibit specific [^{3}H]TCDD binding to any significant extent (IC_{50} > 1500 nM).

To determine the nature of the inhibition of specific [^{3}H]TCDD binding by indolo[3,2-*b*]carbazole, saturation studies were performed in the presence or absence of TCDBF or indolo[3,2-*b*]carbazole, respectively. The results were represented as double-reciprocal plots normalized with respect to protein concentration and analyzed according to Lineweaver and Burk (27) (Fig. 2, A and B, respectively). It is apparent that both TCDBF and indolo[3,2-*b*]carbazole competitively inhibit specific [^{3}H]TCDD binding, suggesting that these two ligands bind to the same site of the receptor as TCDD.

Ball-and-stick models of the ligands investigated here for their binding affinity for the TCDD receptor were built, with appropriate van der Waals radii used for construction of the balls, and studied by means of a computer (as described under "Materials and Methods"). It was obvious from the views featured on the display that all ligands which exhibited a high binding affinity (IC_{50} < 150 nM) for the TCDD receptor in this study were approximately planar. Therefore, the views for plotting were chosen from the display in such a way that the main plane of the molecules coincided with the plotting plane. These plots are shown in Fig. 3. As noted earlier (4) the concept of a 3 × 10 Å rectangle with the centers of the halogen atoms in at least three of its corners as a structural requirement for TCDD receptor-ligands fails to account for the relatively high receptor affinity of some PAH (*e.g.*, benzoflavones). Since the chlorine atoms have relatively large van der Waals radii, we hypothesized that if the van der Waals radii are considered, a more general structure-activity relationship could be established. When the van der Waals radii of all atoms of TCDD are included, the molecule fits into a smallest rectangular envelope of 6.8 × 13.7 Å, as noted by McKinney *et al.* (28). However, the fit of other ligands for the TCDD receptor into this envelope was not discussed (29). In Fig. 3, 6.8 × 13.7 Å envelopes including van der Waals radii are drawn around computer-generated plots of some high-affinity TCDD receptor ligands found in this and other studies. It is apparent that TCDD (A in Fig. 3) and indolo[3,2-*b*]carbazole (C) are approximately isosteric when the van der Waals radii of all atoms are considered. When simple ball-and-stick models (without van der Waals radii) were constructed, we did not observe any structural similarities between these two ligands.

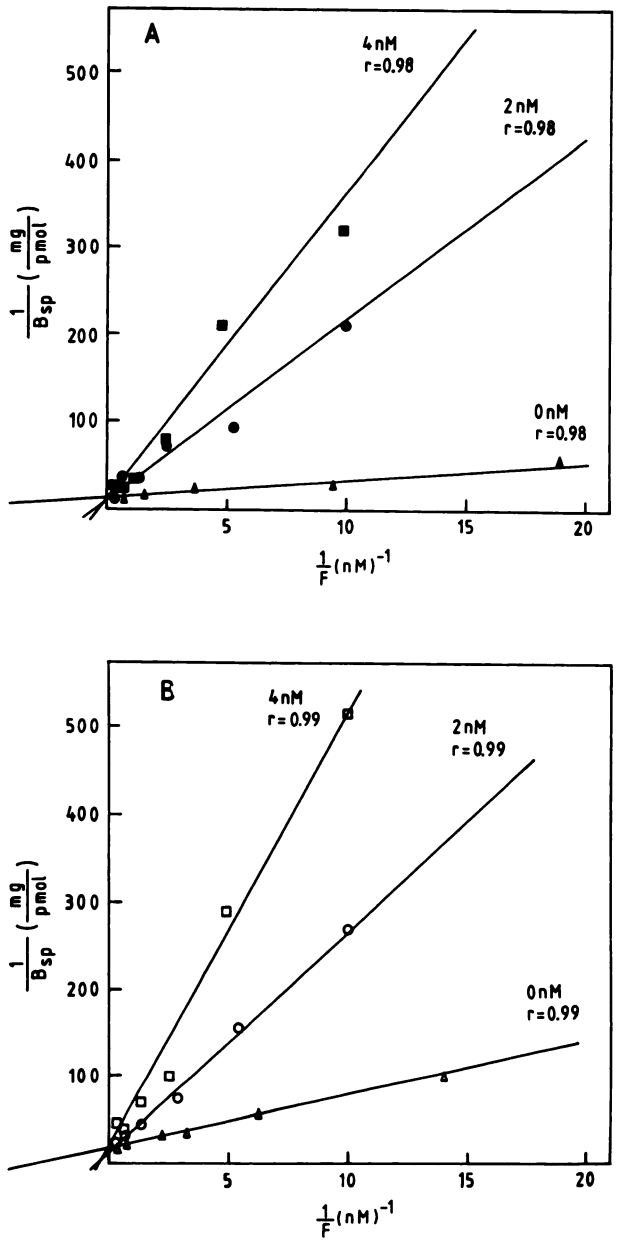

FIG. 2. *Competitive binding of indolo[3,2-b]carbazole and TCDBF to the specific [^{3}H]TCDD-binding sites in rat liver cytosol*

Indolo[2,3-*b*]carbazole (A) or TCDBF (B) was incubated at the indicated concentrations with rat liver cytosol and various amounts of [^{3}H]TCDD, as described under "Materials and Methods," and the results were plotted according to Lineweaver and Burk (27) using linear regression.

Neither were any similarities between TCDD and 5,6-benzoflavone obvious from conventional ball-and-stick models. On the other hand, it is clear from Fig. 3 that the 6.8 × 13.7 Å envelope easily includes 5,6-benzoflavone (D), the most potent unhalogenated TCDD receptor ligand described by Poland and Knutson (4).

The molecular surface area in the plotting plane is not alone sufficient to account for TCDD receptor binding affinity, since indolo[2,3-*a*]carbazole (E) has the same surface area in the plotting plane as its congener indolo[3,2-*b*]carbazole (B), but binds with much lower affinity. A similar situation exists regarding the two ben-

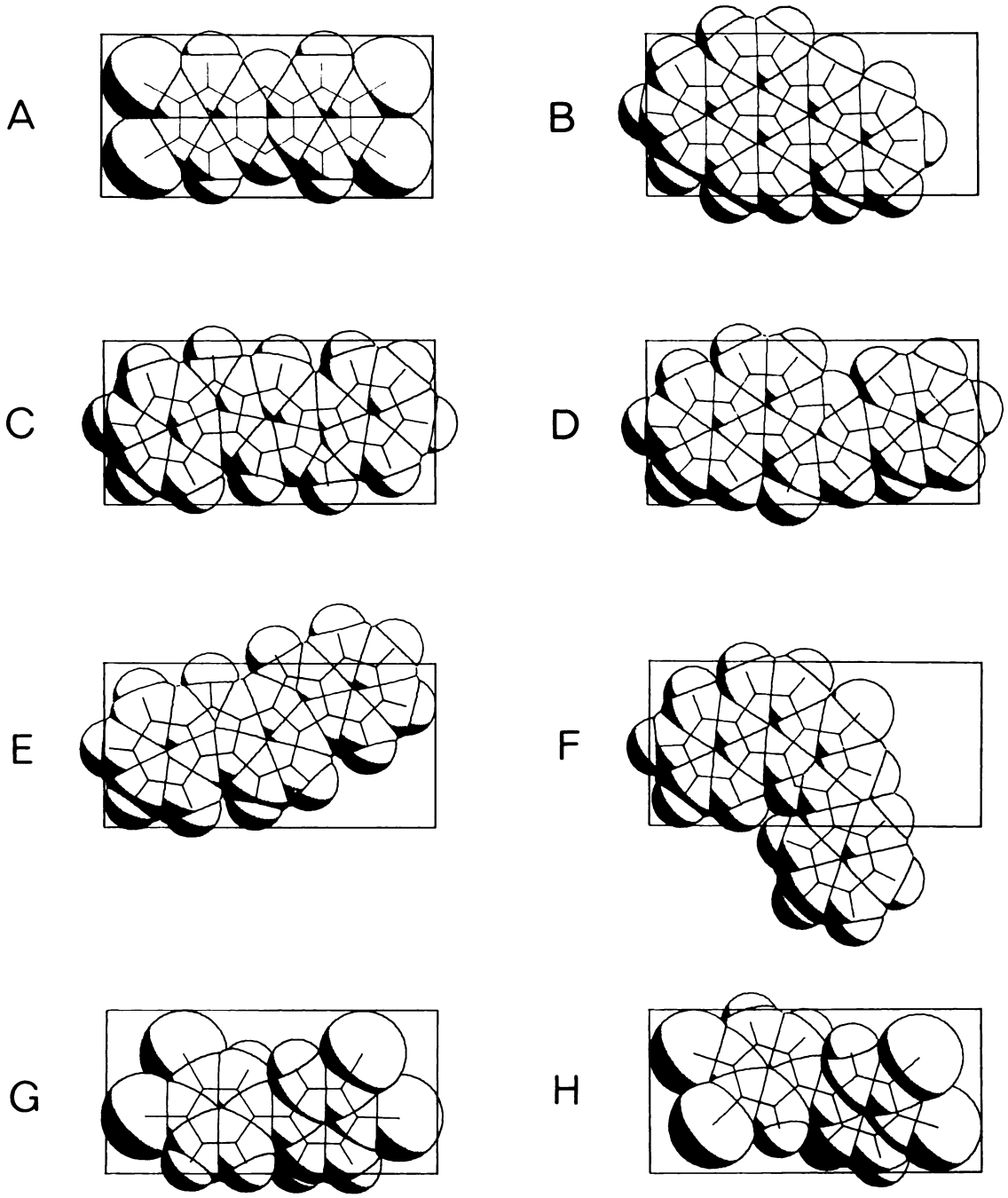

FIG. 3. *Images of TCDD receptor-binding molecules with van der Waals radii included*

The images were built from crystallographic data, and the van der Waals radii according to Pauling (26) were added by means of a computer and plotted, as described under "Materials and Methods." Plots are shown for TCDD (A), benzo[*a*]pyrene (B), indolo[3,2-*b*]carbazole (C), 5,6-benzoflavone (D), indolo[2,3-*a*]carbazole (E), 7,8-benzoflavone (F), and the two possible projections of 3,3′,4,4′-tetrachlorobiphenyl (G, H).

zoflavone isomers, 5,6-benzoflavone (D) and 7,8-benzoflavone (F). Both have the same molecular surface area in the plotting plane, but the latter isomer has a much lower binding affinity for the TCDD receptor. From Fig. 3 it is evident that 7,8-benzoflavone and indolo[3,2-*b*]carbazole are angular and do not fit into the 6.8 × 13.7 Å envelope, when the phenanthrene or carbazole parts of the molecules are given the same orientation as their corresponding linear analogues. Benzo[*a*]pyrene (B; IC_{50} 42.4 ± 9.6 nM) is somewhat shorter and broader and fits less well into the 6.8 × 13.7 Å envelope. This is in line with its lower binding affinity as compared to that of 5,6-benzoflavone (4).

The binding of 3,3′,4,4′-tetrachlorobiphenyl (IC_{50} > 150 nM) to specific [^{3}H]TCDD-binding sites in mouse liver cytosol has been reported to be more than 100 times weaker than that of TCDD (4). In the crystalline structure of chlorinated biphenyls, the phenyl rings are not coplanar. The two rings may rotate, and these compounds may, therefore, occur in two conformations (G

and H), the first of which fits reasonably well into the 6.8 × 13.7 Å envelope. In the two plotted conformations the angle between the rings was set to 40° (23). The indoles with the general structure VIII in Fig. 1 are not large enough to fill more than half of the 6.8 × 13.7 Å envelope (not shown). This is also in line with their low binding affinity.

DISCUSSION

Loub *et al.* (25) have tested the ability of various indoles known to occur in brussels sprouts to induce AHH in the rat liver and small intestine. Following administration by oral intubation, indole-3-carbinol and 3,3′-diindolylmethane were the most potent inducers found. Indole-3-acetonitrile was a weak inducer, whereas ascorbigen, a condensation product of indole-3-carbinol and ascorbic acid occurring in brussels sprouts extracts (13), and indole-3-carboxaldehyde were virtually inactive (25). Arcos *et al.* (29) administered indoles and nitriles, with or without the indole nucleus, intraperitoneally to rats and found 3-indolylmethanol and 3-indolylacetone to be most active inducers of hepatic AHH. Indole, 3-indolylacetonitrile, and dietary L-tryptophan were less active inducers, whereas β-3-indolylethanol and the non-indolic nitriles were inactive (29). It was concluded that the indole nucleus was the critical feature necessary for induction of AHH (29). In apparent conflict with this notion, all 3-substituted indoles we have tested bind weakly to the TCDD receptor.

Since indole-3-carbinol and 3,3′-diindolylmethane apparently bind very weakly to specific TCDD-binding sites in rat liver cytosol as compared to potent AHH inducers such as 5,6-benzoflavone, their AHH-inducing properties earlier observed (2) must be mediated by some other mechanism than direct TCDD receptor binding. Such mechanisms may involve nonreceptor-mediated regulation of microsomal enzymes.

Another possible mechanism is conversion of indoles with low TCDD receptor affinity to high affinity receptor ligands. For example, it has long been known that indole-3-carbinol is very instable in mildly acidic water solutions and then easily forms 3,3′-diindolylmethane (14), which is a substrate for acid-catalyzed chemical synthesis of indolo[3,2-*b*]carbazole (6), the most potent TCDD receptor-binding indole found in this study. This chemical instability of some indoles complicates the interpretation of studies of their microsomal enzyme-inducing capacity.

The TCDD receptor affinity of weakly binding ligands may be more precisely quantitated than in this investigation, as was performed with, *e.g.*, indole-3-carbinol in our earlier study (5). We have refrained from such experiments here, since the value of performing binding studies with high concentrations of competitors is limited; a contamination of the competitor with a minor amount of a TCDD receptor ligand with high binding affinity may alone be responsible for a weak observable binding. A similar risk is also inherent in studies on microsomal enzyme induction by administration of high doses of indoles.

Recent developments in interactive computer graphics have provided methods for molecular modeling. Such methods have been applied to study common topological properties of possible importance for the binding of some inhibitors or substrates to cytochrome P-450 (30). In the cited study the van der Waals surfaces were calculated from crystallographic data using Monte-Carlo techniques (30). In the present study, we have found the van der Waals radii given by Pauling (24) added to the atomic coordinates to be a sufficiently reasonable approximation to obtain conclusive results. However, the radial distribution of the electron density surrounding the centers of chemically bonded atoms is not spherically symmetric nor are the properties of such atoms isotropic (31). In spite of these shortcomings, the hard sphere approximation has been useful in studies of protein conformations, most probably due to the steepness of the repulsive term in the potential function for nonbonded interactions (31).

Other approaches have been applied earlier to rationalize the binding of ligands to the TCDD receptor and the induction of related microsomal enzyme activities. Arcos and Argus introduced the concept of molecular encumbrance area as a measure of the size of planar molecules (32). The molecular incumbrance area is the surface area of the smallest rectangular envelope of a planar molecule drawn proportionally to molecular dimensions, with the van der Waals radii included (32). PAH with an optimal encumbrance area of about 85–150 $Å^2$ induce zoxazolamine hydroxylase (33), an activity correlated with the *Ah* locus (34), which is conceived to control TCDD receptor synthesis (4). However, this approach may be too simplistic to account for TCDD receptor binding of PAH, since PAH with different length/width ratios may have the same encumbrance areas.

The results reported here for some different types of compounds seem to corroborate earlier results for chlorinated hydrocarbons (4), namely that a critical size and planarity of a ligand is required for high-affinity binding ($IC_{50} < 150$ nM) to the TCDD receptor. It has been noted (4) that dioxins that bind with high affinity to the TCDD receptor have at least 3 halogens in lateral positions (2, 3, 7, and 8). These dioxins, as well as some other halogenated receptor ligands, *e.g.*, halogenatd dibenzofurans or biphenyls, fit into a 3 × 10 Å rectangle, with the centers of the halogen atoms in the corners. The length of the rectangle may be near the optimal, since 2,3,6,7-tetrachlorobiphenylene, which is shorter, and 2,3,7,8-tetrachloroanthracene, which is longer, have lower affinity (35) than TCDD for the receptor (least distances between lateral rings in Å: ~1.4–1.5; ~2.4; ~2.3; and K_d in nM: 0.34; 1.4; 0.27, respectively). These three linear ligands have the same type and number of halogens, the same distance between the two chlorine atoms in each pair of lateral halogens, and the same overall molecular thickness.

A limitation of the model of a 3 × 10 Å rectangle as a generalized structure-activity relationship for binding of ligands to the TCDD receptor is that it does not account for the binding of PAH (*e.g.*, benzoflavones (4)) and indoles (*e.g.*, indolo[3,2-*b*]carbazole) to the receptor. The alternative model we have proposed here, a 6.8 × 13.7 Å

envelope including the atomic van der Waals radii of the TCDD receptor-ligand, does not suffer from this limitation. On the contrary, the 6.8 × 13.7 Å envelope seems to give a good fit for the unhalogenated high-affinity binding compounds investigated here, as well as for chlorinated ligands. That the optimal length of the envelope probably is very close to 13.7 follows from the same argument as above for the length of the 3 × 10 Å rectangle when the van der Waals radii are not considered.

An optimal width of the rectangle has not been convincingly shown for any of the concepts. Neither concept accounts for the stronger binding of certain substituted ligands (*e.g.*, 1-CN-TCDD (35)) to the receptor as compared to TCDD. We hope to be able to extend and refine our new concept so that it can be used to predict binding affinity for new molecules. For prediction of the AHH-inducing capacity of TCDD receptor-ligands, the widely differing solubility and rates and routes of metabolism between halogenated and unhalogenated ligands will also have to be taken into account.

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